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RESEARCH ARTICLE

Pulmonary and cardiovascular effects of traffic-related particulate matter: 4-week exposure of rats to roadside and diesel engine exhaust particles

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Abstract

Traffic-related particulate matter (PM) may play an important role in the development of adverse health effects, as documented extensively in acute toxicity studies. However, rather little is known about the impacts of prolonged exposure to PM. We hypothesized that long-term exposure to PM from traffic adversely affects the pulmonary and cardiovascular system through exacerbation of an inflammatory response. To examine this hypothesis, Fisher F344 rats, with a mild pulmonary inflammation at the onset of exposure, were exposed for 4 weeks, 5 days/week for 6 h a day to: (a) diluted diesel engine exhaust (PM_{DEE}), or: (b) near roadside PM ($PM_{2.5}$). Ultrafine particulates, which are largely present in diesel soot, may enter the systemic circulation and directly or indirectly trigger cardiovascular effects. Hence, we assessed the effects of traffic-related PM on pulmonary inflammation and activity of procoagulants, vascular function in arteries, and cytokine levels in the heart 24 h after termination of the exposures. No major adverse health effects of prolonged exposure to traffic-related PM were detected. However, some systemic effects due to PM_{DEE} exposure occurred including decreased numbers of white blood cells and reduced von Willebrand factor protein in the circulation. In addition, lung tissue factor activity is reduced in conjunction with reduced lung tissue thrombin generation. To what extent these alterations contribute to thrombotic effects and vascular diseases remains to be established. In conclusion, prolonged exposure to traffic-related PM in healthy animals may not be detrimental due to various biological adaptive response mechanisms.

Keywords: *Traffic; particulate matter; PM; concentrated particles; pulmonary inflammation; diesel soot; vascular function; procoagulants; air pollution; toxicological*

Introduction

Airborne ambient particulate matter (PM) is considered to play an important role in the adverse health effects associated with air pollution (Brunekreef and Holgate, 2002). Most epidemiological studies have focused on the effects of short-term exposure to air pollutants. In these short-term studies, a clear link was shown between levels of air pollutants and a tendency toward a hypercoagulable state. These associations are, for example, found with both PM_{10} and NO_2 (Baccarelli et al., 2007) or only with the traffic-related gaseous (NO_2 and CO) instead of with PM mass (Rudez et al., 2009). However, several epidemiological studies have associated long-term exposure to the fine fraction of PM ($PM_{2.5}$: PM with an aerodynamic diameter below 2.5 μm) with an increase in pulmonary and cardiovascular morbidity and mortality (Pope et al., 2004; Schikowski et al., 2005). Notably, living close to a busy road over several years has been associated with increased cardiopulmonary mortality

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(Hoek et al., 2002; Gehring et al., 2006). Hence, road traffic, which is a major source of $PM_{2.5}$ in urban areas, could be particularly responsible for the impact of PM exposure on human health (Gauderman et al., 2005; Schikowski et al., 2005; Beelen et al., 2009; Hoffmann et al., 2009).

These epidemiological observations are supported by controlled toxicology studies performed with animals and human volunteers exposed to PM samples from different sites. Animal exposure studies attribute a greater toxicity of PM collected at locations that contain a high proportion of traffic emissions (Lai et al., 2005; Seagrave et al., 2006; Gerlofs-Nijland et al., 2007). Interestingly, it is becoming evident that exposure to traffic-related PM has marked actions on the cardiovascular system, as well as their more well-recognized pulmonary effects (Elder et al., 2007; McCreanor et al., 2007). Short-term exposures to diesel engine exhaust, an important source of $PM_{2.5}$, cause both vascular dysfunction and impaired endogenous fibrinolysis in healthy and compromised volunteers (Mills et al., 2005, 2007). In addition, elevated thrombus formation was shown *ex vivo* after inhalation of diesel engine exhaust (Lucking et al., 2008).

In these studies, mostly fresh generated emission particles were used as a surrogate of PM, which are not necessarily representative of the PM in ambient air. Inhalation of elevated concentrations of ambient air particles collected with different size ranges at different sites by using concentrator technology (Sioutas et al., 1997; Kim et al., 2001a, 2001b) represents a more realistic PM exposure (Lippmann and Chen, 2009). A few hours exposure to $PM_{2.5}$ from urban traffic sites caused an increase in cardiovascular symptoms and in lung toxicity and inflammation in rodents and volunteers (Gong et al., 2003; Cassee et al., 2005; Kleinman et al., 2005; Lippmann et al., 2005a; Kooter et al., 2006; Araujo et al., 2008; Ying et al., 2009). This also suggests a major contribution of traffic-related particles to the biological effects associated with PM. Therefore, these studies, in which relatively high PM exposure levels were applied, will be useful in understanding the impact of episodic PM exposure on human health.

At present, only a few publications, all from the same well-conducted study in New York, describe the impact of prolonged exposure on normal and susceptible (i.e. mimicking a human disease) rodents at lower concentration, more environmentally relevant levels (Chen and Hwang, 2005; Chen and Nadziejko, 2005; Hwang et al., 2005; Lippmann et al., 2005b; Sun et al., 2005, 2008; Veronesi et al., 2005). The most striking results were seen on the cardiovascular system with altered vasomotor tone, induced vascular inflammation, and potentiated atherosclerosis both in Sterling Forest (Sun et al., 2005) and more traffic-influenced Manhattan (Ying et al., 2009).

In order to investigate the contribution of traffic to the long-term effect of particles, we performed a series of experiments in which we exposed rats to filtered air, to diluted diesel engine exhaust (rich in ultrafine particles), and to $PM_{2.5}$ derived from a nearby very busy freeway. Prior to the PM exposures, a minor lung inflammation was induced by exposing the rats to ozone. We hypothesized that prolonged (i.e. 4-week) exposure to traffic-derived $PM_{2.5}$ exacerbates the existing inflammatory reaction, which could result in an induction of oxidative stress with subsequent effects on the pulmonary and cardiovascular system. In order to verify this hypothesis, a comprehensive analysis of markers for pulmonary (oxidative stress, cytotoxicity, inflammation) and cardiovascular (coagulation, fibrinolysis, endothelial damage, thrombogenicity, heart inflammation, aorta contractibility) effects due to exposure to traffic-derived PM was performed.

Methods

Animals

Male SPF F344 (DUCRL) rats were obtained from Charles River (Sulzfeld, Germany). The rats were housed in macrolon type III cages with a room temperature maintained at 22 ± 2°C, relative humidity at 40–70%, and a 12-h light/dark cycle. Rats were allowed access to a cereal-based rodent diet (SMR-A; Hope Farms, Woerden, The Netherlands) and tap water via drinking bottles ad libitum during non-exposure periods. Exposure started after 7 days of acclimatization.

Experimental design

A total of three experiments were conducted using different types of PM exposure (Gerlofs-Nijland et al., 2009a). At day 0, all rats were exposed (whole body) for 12 h to 0.4 ppm ozone (Marra and Rombout, 1990) to initiate a minor inflammation in the lung (Cassee et al., 2005). After the initial ozone exposure, the animals were transferred to RIVM's mobile exposure laboratory (MAPCEL) and subsequently exposed for 4 weeks (5 days per week, 6 h per day) to diesel engine exhaust (PM_{DEE}) or to concentrated ambient particles with an aerodynamic diameter <2.5 μm ($PM_{2.5}$ or also known as CAPs) near a busy roadside at Utrecht, The Netherlands (Figure 1). The $PM_{2.5}$ roadside study was repeated once due to the inherent variability of the mass and composition of ambient PM.

PM_{DEE}

Exposure was performed using a 35 KVA diesel generator (Bredenoord, Apeldoorn, The Netherlands) under idling conditions. The animals (n = 15/group) were exposed to 150 μg/m^3 PM_{DEE} diluted with clean conditioned air.

$PM_{2.5}$ roadside

Rats were exposed to increased levels of $PM_{2.5}$ using the Versatile Aerosol Concentration Enrichment Systems (VACES) (Kim et al., 2001a, 2001b) with a theoretical enrichment factor of 20 and at an output flow rate directed into the nose-only system of 20 LPM. The MAPCEL was placed close to (15 m), and east of a major roadside (A2; Utrecht–Amsterdam, The Netherlands), with prevailing westerly winds, used by 160,000 cars and trucks per day.

Control animals were exposed to filtered, purified air with the same temperature and relative humidity as the test atmospheres. All rats were nose-only exposed using novoplast tubes T (Münster AG, Muttenz, Switzerland) in nose-only exposure chambers. One week before exposure, animals were trained

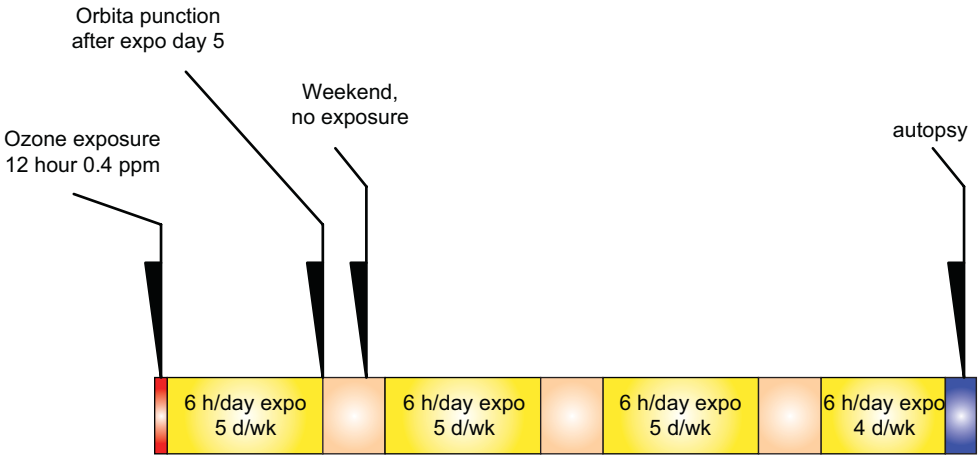

Figure 1. Experimental exposure design.

in nose-only tubes to reduce the stress of the restraint (3 days, 1 h per day). Immediately after the exposures, the animals were returned to their housing facilities.

Blood (1 ml) was obtained after the first week of exposure (directly after the fifth exposure day; Figure 1) by orbital puncture under Brevimethal anaesthesia (50 mg/kg bodyweight, intramuscularly) to measure fibrinogen, von Willebrand factor (vWF), plasminogen activator inhibitor (PAI)-1, and CC16.

Based on the initial findings, additional parameters were investigated, i.e. vascular function, measurement of cytokines in cardiac tissue, and tissue factor (TF) activity and thrombin generation in lung tissue in one of the two roadside experiments to gain more insight in a possible biological mechanism.

Necropsy was performed on the day after the last exposure day (Figure 1). Experiments were approved by the Animal Ethics Committee (IUCAC) of the Dutch National Vaccine Institute (NVI, Bilthoven, The Netherlands).

Characterization of the test atmospheres

A condensation particle counter (CPC model 3022A; TSI, St. Paul, MN) was used to determine the particle number concentration in the inlet of the exposure chamber. The mass concentration was measured continuously in the inlet of the exposure chamber during the exposure with a nephelometer (DATARAM 2000; MIE, Billerica, MA). In the $PM_{2.5}$ roadside experiments, the particle number and mass concentration were measured both before the VACES inlet and after the VACES. The time-integrated PM concentrations were also measured in the inlet of the exposure chamber by means of collection on three 47-mm filters placed in parallel, two polytetrafluoroethylene (PTFE; Teflon R2PJ047; Pall Corp., Ann Arbor, MI), and one quartz filter (QMA; Whatman Int Inc, Maidstone, England). A carbon sampler tube (Anasorb CSC Lot 2000; SKC Inc., Eighty Four, PA) was placed downstream of one of the PTFE filters at the outlet to collect the volatile organic components (VOCs). One set of PTFE filters and a carbon sampler tube were used for each exposure week. Carbon monoxide (ML 9830 CO; Lear Siegler, Englewood, CO), sulfur dioxide (model 43A; Thermo Environmental Instruments, Franklin, MA), and nitrogen oxides (model 42W; Thermo Environmental Instruments) were measured in the PM_{DEE} mixing chamber or at the inlet of the VACES. In the PM_{DEE} experiment, a Scanning Mobility Particle Sizer (SMPS, DMA model 3071 + CPC model 3022A; TSI) was used to measure the particle size distribution (mean diameter and geometric standard deviation) every hour in the inlet of the exposure chamber. The weekly time-integrated particle size mass distribution was measured at the inlet of the VACES with an eight-stage Micro Orifice Impactor (model No. 100; MSP Corporation, Minneapolis, MN). Temperature and relative humidity were recorded once every 5 min in the exposure chamber and control exposure chamber and recorded every 30 min in the inlet of the exposure chamber. The activated carbon samplers were analyzed using GC-MS (RIVM, Bilthoven, The Netherlands) to determine the VOC concentrations.

Necropsy

The day after the final test atmosphere exposure, the rats were anesthetized with a mix of Ketamine and Rompun: 100 mg/kg of Ketamine (Aesculaap, Boxtel, The Netherlands) and 1 mg/kg Rompun (Bayer, Leverkusen, Germany). A cannula was inserted in the trachea. The abdomen was opened and a minimum of 6 ml blood was sampled through the abdominal aorta. The chest was opened and the lungs were perfused (pressure 30 cm H_2O) with saline to remove the blood from the lung using a cannula placed through the right heart chamber into the pulmonary artery. The left bronchus was clamped and the left lung was cut just behind the clamp. The left lung was weighed and fixed for 1 h under a constant pressure of 20 cm H_2O using 4% phosphate-buffered formaldehyde. The right lung was used for bronchoalveolar lavage fluid (BALF) collection by three lavages of sterile saline (27 ml/kg body weight). The heart was dissected, split into the right and left side and frozen in liquid N_2. The descending thoracic aorta was dissected and immediately placed in Krebs buffer for organ bath measurements.

Bronchoalveolar lavage analyses

The collected BALF was centrifuged at 400*g*, 4°C, for 10 min. The cell-free fluid from the lavage was used for assessment of lactate dehydrogenase (marker for cytotoxicity),

N-acetylglucosaminidase (macrophage activation), alkaline phosphatase (type II cell damage), and the levels of Clara-cell 16 protein (CC16, lung cell damages), reduced glutathione and oxidized glutathione (GSH and GSSG, respectively), albumin and total protein levels (increased permeability of the alveolar–capillary barrier), inflammatory mediators interleukin 6 (IL-6), and tumor necrosis factor (TNF)-α were determined as previously described (Cassee et al., 2005; Gerlofs-Nijland et al., 2005). Heme-oxygenase-1, a marker of oxidative stress, was determined using a commercially obtained reagent kit (Roche Nederland B.V, Mijdrecht, The Netherlands). The BALF pellet was resuspended in saline and used for total cell counts as well as preparation of cytospins for cell differential counts as previously described (Gerlofs-Nijland et al., 2005).

Hematological analyses

Plasma levels of fibrinogen and CC16 were determined as previously described (Cassee et al., 2005; Gerlofs-Nijland et al., 2009b). vWF was measured by enzyme-linked immunosorbent assay (ELISA; American Diagnostica Inc., Stamford, US). Levels of tissue plasminogen activator, total antigen, and active PAI-1 were measured in citrated plasma by ELISA (Innovative Research, Dearborn, M). Cell differentials were determined in EDTA (K3) (Terumo Europe N.V., Leuven, Belgium) anticoagulated blood in an H1-E multispecies hematology analyzer (Bayer B.V., Mijdrecht, The Netherlands). The following parameters were measured: white and red blood cell concentrations (WBC and RBC, respectively), hemoglobin, and platelet (PLT) concentrations, the mean platelet volume, and the hematocrit value. In addition, mean corpuscular volume, mean cell hemoglobin, mean cell hemoglobin concentration, red blood cell distribution width, mean platelet component and hemoglobin distribution width were provided.

Pathology

The left lung was embedded in paraffin after fixation with formaldehyde. Tissues were cut into 5-μm slices and slides were stained with hematoxylin and eosin before light microscopic examination. Slides were screened for pathological changes as a result of the exposure. The pathological lesions and inflammation were semi-quantitatively and blindly scored as absent, minimal, slight, moderate, marked, or strong.

Vascular function

Ex vivo endothelial function and vascular responses were measured in isolated thoracic aortic rings by a modified method of Bagate et al. (2004) and Miller et al. (2009). Segments of thoracic aorta (~5 mm length) were cleaned of connective tissue and mounted in organ baths in Krebs buffer bubbled with 5% CO_2/95% O_2 at 37°C. A baseline tension of 14.7 mN was gradually applied over 10 min and vessels were allowed to equilibrate for a further 30 min.

Vessel viability was confirmed by a contractile response on addition of 80 mM KCl, repeated three times. Concentration–response curves to phenylephrine (PE; 1 nM to 10 μM) were obtained and a concentration that produced 80% maximum contraction (0.1–1 μM) was chosen for each individual rat aortic ring. Following contraction, cumulative concentration–response curves were obtained for acetylcholine (ACh; endothelium-dependent vasodilator; 1 nM to 10 μM), sodium nitroprusside (SNP; endothelium-independent nitric oxide donor; 0.1 nM to 1 μM), and isoprenaline (ISP) or verapamil (endothelium- and nitric oxide–independent vasodilators; 1 nM to 10 μM). At least 30-min washout was allowed before application of subsequent drugs.

Analyses of cardiac tissue

Cytokine mRNA expression

The frozen right heart halves were homogenized in lysis buffer and total RNA isolated using a "Absolutely RNA™ RT-PCR Miniprep kit" (Stratagene, La Jolla, CA). mRNA in each sample was reverse-transcribed into cDNA on a PCR system 2400 (Perkin Elmer, Groningen, The Netherlands) by using a High Capacity cDNA Archive Kit from Applied Biosystems (Life Technologies Corporation, Carlsbad, CA). Quantitative real-time (QRT) PCR was performed on triplicate samples, with 18S rRNA as an internal control, using the Applied Biosystems 7500 Real-Time PCR System, with pre-designed TaqMan Gene Expression Assays (IL-6, Rn00561420_m1; IL-1β, Rn00580432_m1; TNF-α, Rn00562055_m1; 18S, Hs99999901_s1) and TaqMan Universal PCR Master Mix. The expression of each gene within each sample was normalized against 18S rRNA and expressed relative to a heart tissue sample from one of the control rats using the formula $2^{-(\Delta\Delta C_t)}$ in which $\Delta\Delta C_t = (C_t$ mRNA – C_t 18S rRNA) sample – (C_t mRNA – C_t 18S rRNA) sample control rat.

Phosphorylation of mitogen-activated protein kinases

Right heart halves were homogenized in lysis buffer (20 mM Tris–HCl pH+7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.4 mM Na-pyrophosphate, 1.0 mM orthovanadate, 1 mM NaF, 21 μM leupeptin, 1.5 μM aprotinin, 15 μM pepstatin A, and 1% Triton-X) and examined by western analysis. Protein concentration in the samples was determined by using the Bio-Rad DC Protein Assay (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands). Proteins (12.5–25 μg/well) from the homogenized heart tissue samples were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes. To ensure that the protein levels of each well were equal, Ponceau-staining was used for loading control. The membranes were then probed with antibodies for the respective phosphorylated kinases (p-ERK1/2, p-JNK1/2, p-p38) prior to incubation with horseradish peroxidase–conjugated secondary antibodies. The blots were developed using the Super-Signal® West Dura chemiluminescence system (Perbio Science Nederland B.V., Etten-Leur, The Netherlands) according to the manufacturer's instructions. Finally, the membranes were stripped by incubation for 15 min at room temperature with Mild Antibody Stripping Solution® from Chemicon International (Temecula, CA), and re-probed with antibodies against total mitogen-activated protein kinase (MAPK) proteins (ERK1/2, JNK, p38). Optical quantification of the protein bands were performed by using the KODAK 1D Image Analysis Software.

TF activity and thrombin generation in lung tissue

TF activity and tissue-specific thrombin generation by means of the Calibrated Automated Thrombogram (Thrombinoscope BV, Maastricht, The Netherlands) were determined in lung tissue homogenates as described previously (Frederix et al., 2008). Briefly, thrombin generation was measured in the presence of a final concentration of 5 pM TF and 4 μM phospholipids (PL, at 20:20:60 mol% PS:PE:PC) after addition of lung homogenates in human plasma and alternatively measurements were also implemented in the absence of both TF and PL. All TG results were normalized and expressed as percentage of normal pooled, PLT-poor plasma which was prepared from at least 80 healthy volunteers (Spronk et al., 2008).

Statistical analysis

Data are expressed as mean ± standard deviation (SD) or standard error of mean (SEM). Vascular responses are expressed as percentage of the maximal contraction to PE, where positive values represent vasodilatation and 100% vasodilatation represents a complete abolition of PE-induced tone. The outcomes of the BALF, blood analyses, TF activity, and thrombin generation were compared using an unpaired Student's *t*-test. Statistical comparisons of vasodilator curves were carried out using two-way analysis of variance (ANOVA), or unpaired Student's *t*-test for comparisons of EC_{50} and maximum responses (estimated following linear regression of individual curves using GraphPad Prism V4.0b). $P < 0.05$ was accepted as statistically significant.

Results

Ozone exposure

A separate group of 10 animals was used to confirm that ozone exposure induced a minor lung inflammation. At 24 h after the ozone exposure, there was a significant increase in lung permeability, as shown by elevated protein (487 ± 141 compared to control levels of 159 ± 49 mg/l; $P < 0.001$) and albumin (248 ± 99 mg/l versus 50 ± 14 mg/l in control group; $P < 0.001$) levels in BALF. Ozone exposure also increased the percentage of polymorphonuclear neutrophils in the alveolar region by approximately 2.5% (3.05 ± 2.55% versus 0.65 ± 0.95% for control), although this increase did not reach statistical significance ($P > 0.05$).

Exposures characteristics

Diesel engine exhaust

The PM_{DEE} exposures were performed at an overall average particle mass of 174 ± 15 μg/m³ (Table 1). The average particle size (geometric median diameter) was 76 nm with a geometric standard deviation of 1.95 nm as measured by SMPS, with an average particle number concentration of 434,000/cm³. During the first exposure week, the carbon sampler was used only for 1 day and the amount of VOC measured was 564 μg/m³. In addition, the concentrations of gaseous pollutants CO, NO, NO_2, and NOx were measured, with mean concentrations of 3050, 1671, 918, and 2589 μg/m³, respectively. Levels of VOC during the last three exposure weeks could not be measured due to an overload in the carbon sampler tubes.

$PM_{2.5}$ roadside #1

During the first $PM_{2.5}$ roadside study, the overall average particle mass was 485 ± 150 μg/m³ (Table 1). The average particle number concentration was 312,000/cm³ with a mean aerodynamic particle size of 1.04 μm and geometric standard deviation of 0.31 (measured by multiplicity of infection (MOI) before the VACES). The mean VOC content measured was 254 μg/m³, which was mainly driven by high levels of VOC (820 μg/m³) during the first week of exposure. These appeared to be caused by high amounts of heptane, most probably due to a two-stroke engine used for lawn mowing activities nearby. The concentrations of gaseous pollutants NO, NO_2, and NOx were 56, 71, and 127 μg/m³, respectively.

Table 1 - Particle exposure characteristics of diesel engine exhaust and concentrated ambient particles near a roadside.

Experiment	Week no	Mass	Number	CO	NO	NO_2	NOx	VOC	Inorganics	MMAD†
		μg/m³	# 10^5/cm³	μg/m³	μg/m³	μg/m³	μg/m³	μg/m³	μg/m³	μm
	1	160	4.83	3515	2058	1128	3186	564	nd	nd
	2	162	4.44	3131	1671	937	2608	897‡	nd	0.29
PM_{DEE}	3	191	4.18	2945	1571	841	2413	5416‡	nd	0.24
	4	182	3.89	2689	1397	765	2162	4068‡	nd	0.17
	Average	174	4.34	3050	1671	918	2589	564		0.23
	1	484	3.72	nd	72	84	156	820	nd	1.18
	2	284	3.51	nd	65	76	141	70	nd	0.99
$PM_{2.5}$	3	528	1.71	nd	22	42	65	45	nd	1.01
roadside #1	4	643	3.53	nd	69	82	151	80	nd	0.97
	Average	485	3.12	—	56	71	127	254		1.04
	1	200	2.46	nd	82	48	130	6	44	1.46
	2	199	2.63	nd	87	71	158	6	60	1.53
$PM_{2.5}$	3	224	2.32	nd	85	59	144	49	46	2.13
roadside #2	4	232	1.84	nd	70	63	133	13	81	0.95
	Average	214	2.31	—	81	61	142	19	58	1.52

PM, particulate matter; DEE, diesel engine exhaust; Inorganics, sum of sulfate, nitrate, chloride and sodium; VOC, volatile organic components; MMAD, Aerodynamic particle size measured by MOI; nd, not determined

‡Unreliable outcomes due to overload carbon samplers, those values are not included in the average VOC content.

$PM_{2.5}$ roadside #2

The overall average particle mass in the second $PM_{2.5}$ roadside study was 214 ± 17 μg/m³. The average particle number concentration was 231,000/cm³ with an associated aerodynamic mean particle size of 1.52 μm and geometric standard deviation of 0.23 (measured by MOI before the VACES). The VOC content was 19 μg/m³ with NO, NO_2, and NOx concentrations of 81, 61, and 142 μg/m³, respectively.

BALF analyses

Prolonged exposure to PM_{DEE} or $PM_{2.5}$ near a roadside did not induce a detectable inflammatory response in healthy rats. The number of MN in BALF was not significantly increased after 4 weeks of exposure to $PM_{2.5}$ roadside or PM_{DEE}; nor were there any changes in the pro-inflammatory cytokines TNF-α and IL-6 (Table 2). Although some parameters (e.g. TNF-α, protein) showed strong differences with higher values after exposure to roadside $PM_{2.5}$, the only statistically significant change was an increase in BALF CC16 after exposure to $PM_{2.5}$ in the second roadside study (9.47 ± 1.14 versus 8.49 ± 1.21 in the control group; $P < 0.05$; Table 2). Notably, protein and albumin levels in BALF were significantly higher in all animals that were transported to our field location near the freeway compared to those that were exposed in our laboratory at the RIVM. Apart from the fact that batch-to-batch variation among the groups of animals that we received from the breeder cannot be excluded, the only other explanation is that the transport from the field location to the lab might have resulted in increased stress and increased baseline values of the noted parameters. Since most of the other parameters that we have assessed were not to be affected in a similar manner, and we performed the (statistical) comparisons only within each of the three experiments, conclusions were not affected by this unexpected phenomenon.

Hematological analyses

Prolonged PM_{DEE} exposure resulted in significantly reduced numbers of WBCs, lymphocytes, and basophilic granulocytes (Table 3). On the other hand, neither $PM_{2.5}$ roadside exposure induced any significant changes in blood parameters, although a small decrease of lymphocyte number was observed in the second $PM_{2.5}$ roadside study. A reduction in the blood vWF levels was observed 4 weeks after exposure to PM_{DEE} (112.2 ± 34.2 mU/ml versus 132.5 ± 13.2 mU/ml in the control group; $P < 0.05$; Table 3).

Lung pathology

The lungs of the animals exposed to PM_{DEE} showed a number of minor changes including perivascular and peribronchial inflammatory cell infiltrates and mononuclear inflammatory cells (lymphocytes). The number of alveolar macrophages was generally low and there was no infiltration of neutrophilic or eosinophilic leukocytes. Although the incidence of a few changes was slightly increased, there were no changes that distinctly and convincingly could be related to PM_{DEE} exposure.

Roadside $PM_{2.5}$ exposures resulted in a diffuse accumulation of alveolar macrophages in the lungs of all animals, albeit in low numbers. There was no infiltration of neutrophilic or eosinophilic leukocytes. Diffuse macrophage accumulation tended to be slightly more severe in $PM_{2.5}$-exposed rats compared to rats exposed to filtered air (though not statistically significant). Alveolar macrophages of $PM_{2.5}$-exposed rats contained small dark-stained phagocytized particles, which were

Table 2. Parameters in bronchoalveolar lavage fluid after exposure to PM from diesel engine exhaust or concentrated ambient particles near a roadside.

		PM_{DEE}		$PM_{2.5}$ roadside #1		$PM_{2.5}$ roadside #2	
BALF		Control	PM exposure	Control	PM exposure	Control	PM exposure
Parameter	Unit	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Macrophages	%	95.5 ± 1.5	95.0 ± 2.6	96.3 ± 1.2	96.4 ± 3.7	98.3 ± 0.8	98.6 ± 0.6
PMN	%	3.18 ± 1.31	3.33 ± 1.88	2.10 ± 1.05	2.25 ± 2.93	0.98 ± 0.40	0.71 ± 0.37
Lymphocytes	%	1.18 ± 0.62	1.45 ± 0.89	1.47 ± 0.87	1.23 ± 0.80	0.77 ± 0.47	0.63 ± 0.40
Total cells	#×10⁶	0.91 ± 0.31	1.058 ± 0.3	0.63 ± 0.18	0.57 ± 0.17	0.67 ± 0.16	0.65 ± 0.23
Macrophages	#×10⁶	0.87 ± 0.3	1.006 ± 0.28	0.61 ± 0.18	0.55 ± 0.17	0.66 ± 0.16	0.64 ± 0.23
PMN	#×10⁶	0.03 ± 0.02	0.036 ± 0.03	0.01 ± 0.01	0.01 ± 0.02	0.006 ± 0.003	0.005 ± 0
Lymphocytes	#×10⁶	0.01 ± 0	0.016 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.005 ± 0.004	0.004 ± 0
HO-1	ng/mL	0.09 ± 0.04	0.11 ± 0.04	0.31 ± 0.08	0.32 ± 0.08	0.05 ± 0.05	0.03 ± 0.04
TNF-a	ng/mL	16.3 ± 5.9	16.6 ± 10.7	29.1 ± 7.2	30.4 ± 6.9	137.0 ± 31.8	133.5 ± 32.5
IL-6	μg/mL	57.2 ± 12.3	61.5 ± 26.6	87.3 ± 19.2	90.0 ± 20.2	42.9 ± 13.3	45.5 ± 12.0
CC-16	μg/mL	5.08 ± 1.44	4.76 ± 2.05	7.02 ± 1.90	7.45 ± 2.84	8.49 ± 1.21	9.47* ± 1.14
ALP	U/L	36.3 ± 11.1	35.5 ± 16.9	38.4 ± 13.0	40.8 ± 12.5	44.3 ± 10.1	44.3 ± 7.4
LDH	U/L	108 ± 29	112 ± 40	180 ± 93	174 ± 57	106 ± 9	92 ± 10
Protein	mg/L	183 ± 36	177 ± 40	282 ± 156	302 ± 103	359 ± 63	363 ± 85
Albumin	mg/L	117 ± 24	115 ± 31	184 ± 108	209 ± 77	184 ± 38	185 ± 56
NAG-B	U/L	1.06 ± 0.38	1.34 ± 0.43	2.91 ± 0.64	3.38 ± 0.57	n.d. ± n.d.	n.d. ± n.d.
Total glutathion	μmol/L	0.82 ± 0.44	1.27 ± 1.13	1.52 ± 0.93	1.48 ± 0.56	1.46 ± 0.90	1.70 ± 0.90
GSSG	μmol/L	0.14 ± 0.13	0.13 ± 0.18	0.45 ± 0.26	0.41 ± 0.22	0.64 ± 0.42	0.55 ± 0.21
GSH	μmol/L	0.55 ± 0.36	1.04 ± 0.96	0.74 ± 0.87	0.67 ± 0.52	0.37 ± 0.47	0.69 ± 0.67

*$P < 0.05$ compared to experimental control

not observed in controls and should be therefore considered as a result of the PM exposure.

Focal subpleural accumulations of alveolar macrophages accompanied by thickened alveolar septa occurred in animals exposed to all three PM test atmospheres. However, the incidence was statistically significantly increased in roadside $PM_{2.5}$-exposed rats ($P < 0.05$, Fisher's exact test).

Because no adverse, treatment-related effects were detected, no actual data on the pathological analysis are presented here.

Vascular function

In isolated rat aortic rings, the vasodilator PE caused a concentration-dependent contraction (Figure 2). The response to PE was not different between control animals and PM_{DEE}-exposed animals or control animals and animals exposed to roadside $PM_{2.5}$ ($P > 0.05$ for all, two-way ANOVA; $n = 4$–6). ACh, SNP, and ISP all caused concentration-dependent relaxation of PE-contracted tissue. Responses in tissue from PM_{DEE}-exposed animals or roadside $PM_{2.5}$-exposed animals were not different from their respective controls ($P > 0.05$ for all). In light of these results, organ bath analysis was not performed in the repetition of the $PM_{2.5}$ roadside study.

Cardiac tissue

Samples of heart tissue from control and exposed rats were examined with regard to expression of IL-1β, TNF-α, and IL-6 mRNA as well as to phosphorylation of MAPKs. Neither the expression of mRNA for these cytokines, nor the phosphorylation of the investigated MAPKs differed between control and PM-exposed animals (data not shown).

TF activity and thrombin generation

Lung TF activity was significantly decreased after exposure to PM_{DEE} (control 368 ± 61 pM versus PM_{DEE} 218 ± 51 pM; $P = 0.009$; Figure 3) and slightly diminished after $PM_{2.5}$ roadside exposure (control 348 ± 19 pM versus $PM_{2.5}$ 310 ± 34 pM; $P = 0.047$; Figure 3). Furthermore, partly coherent changes to lung TF activity were observed in thrombin generation, since the lag time is prolonged for exposure to PM_{DEE} (control 86% ± 3% versus PM_{DEE} 90% ± 3%; $P = 0.039$; Figure 3) whereas the lag time is shortened by exposure to $PM_{2.5}$ (control 97% ± 2% versus $PM_{2.5}$ roadside 91% ± 3%; $P = 0.015$; Figure 3) for the latter measured both with and without the addition of TF and phospholipids. Overall thrombin generation, as depicted by the ETP, was not altered upon long-term exposure to traffic-related PM: ETP control 51% ± 4% versus PM_{DEE} 46% ± 3% ($P = 0.231$; Figure 3) and control 58% ± 3% versus $PM_{2.5}$ roadside 51% ± 5% ($P = 0.383$; Figure 3). In addition, analysis of lung tissue thrombogenicity in the absence of additional TF and phospholipids demonstrated an overall decreased lung-induced thrombin generation for long-term exposure to PM_{DEE} (ETP: control 38% ± 4% versus PM_{DEE} 28% ± 3%; $P = 0.027$), whereas no changes were observed after long-term exposure to $PM_{2.5}$ (38% ± 3% versus $PM_{2.5}$ roadside 32% ± 3%; $P = 0.197$). The attenuation of lung tissue–induced

Table 3. Parameters in blood after exposure to diesel engine exhaust or concentrated ambient particles near a busy roadside.

		PM_{DEE}		$PM_{2.5}$ roadside #1		$PM_{2.5}$ roadside #2	
BALF		Control	PM exposure	Control	PM exposure	Control	PM exposure
Parameter	Unit	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
4 Weeks							
RBC	x 10^{12}/L	8.73 ± 0.36	8.65 ± 0.23	8.62 ± 0.23	8.59 ± 0.28	8.37 ± 0.20	8.41 ± 0.30
HGB	mmol/L	9.20 ± 0.39	9.02 ± 0.23	8.90 ± 0.31	8.93 ± 0.32	8.81 ± 0.21	8.82 ± 0.30
HCT	L/L	0.416 ± 0.016	0.412 ± 0.015	0.395 ± 0.015	0.396 ± 0.015	0.392 ± 0.010	0.393 ± 0.018
HDW	mmol/L	1.832 ± 0.079	1.855 ± 0.052	1.954 ± 0.071	1.930 ± 0.111	1.795 ± 0.101	1.806 ± 0.111
PLT	x 10^9/L	448 ± 57	431 ± 43	476 ± 89	462 ± 158	640 ± 60	671 ± 88
MPC	g/dL	22.59 ± 0.79	22.07 ± 0.93	22.87 ± 0.83	22.97 ± 0.75	22.81 ± 0.75	23.02 ± 0.61
WBC	x 10^9/L	3.69 ± 1.01	2.91*±0.5	3.34 ± 0.92	3.60 ± 1.11	4.23 ± 0.89	3.68 ± 0.82
PMN	x 10^9/L	0.77 ± 0.26	0.67 ± 0.18	0.76 ± 0.25	0.86 ± 0.28	0.68 ± 0.18	0.67 ± 0.18
Lymphocytes	x 10^9/L	2.78 ± 0.71	**2.14* ± 0.43**	2.47 ± 0.73	2.61 ± 0.85	3.36 ± 0.68	**2.85† ± 0.64**
Basophils	x 10^9/L	0.008 ± 0.005	**0.002* ± 0.003**	0.026 ± 0.013	0.028 ± 0.017	0.026 ± 0.019	0.022 ± 0.007
PMN	%	20.6 ± 2.1	23.1 ± 5.6	23.1 ± 5.2	24.2 ± 5.7	16.2 ± 2.6	18.14 ± 2.74
Lymphocytes	%	75.86 ± 2.55	73.37 ± 5.79	74.02 ± 5.11	72.44 ± 6.11	79.58 ± 3.31	77.49 ± 2.79
Basophils	%	0.196 ± 0.069	0.132 ± 0.078	0.746 ± 0.263	0.778 ± 0.297	0.549 ± 0.307	0.583 ± 0.137
vWF	mU/mL	133 ± 13	**112* ± 34**	136 ± 29	124 ± 41	163 ± 22	153 ± 26
PAI-1	ng/mL	0.15 ± 0.12	0.24 ± 0.15	0.16 ± 0.1	0.22 ± 0.11	0.16 ± 0.13	0.20 ± 0.19
tPA tot	ng/mL	0.13 ± 0.04	0.12 ± 0.04	0.13 ± 0.05	0.13 ± 0.04		
Fibrinogen	mg/mL	1.74 ± 0.79	1.77 ± 0.67	1.52 ± 0.49	1.46 ± 0.65	2.63 ± 0.50	2.99 ± 1.16
CC16	ng/mL	30.3 ± 21.1	25.3 ± 14.1	19.5 ± 13.2	15.6 ± 10.1	25.9 ± 2.7	27.0 ± 4.5
Day 6							
vWF	mU/mL	193 ± 83	165 ± 78	198 ± 44	222 ± 21	137 ± 20	120 ± 37
PAI-1	ng/mL			0.27 ± 0.09	0.21 ± 0.12	0.37 ± 0.17	0.26 ± 0.19
CC16	ng/mL					25.1 ± 2.8	26.4 ± 4.3
Fibrinogen	mg/mL	1.24 ± 0.48	1.27 ± 0.76	1.05 ± 0.33	0.99 ± 0.26	2.44 ± 0.45	2.69 ± 0.71

*P<0.05 compared to experimental control; †P=0.05 compared to experimental control.

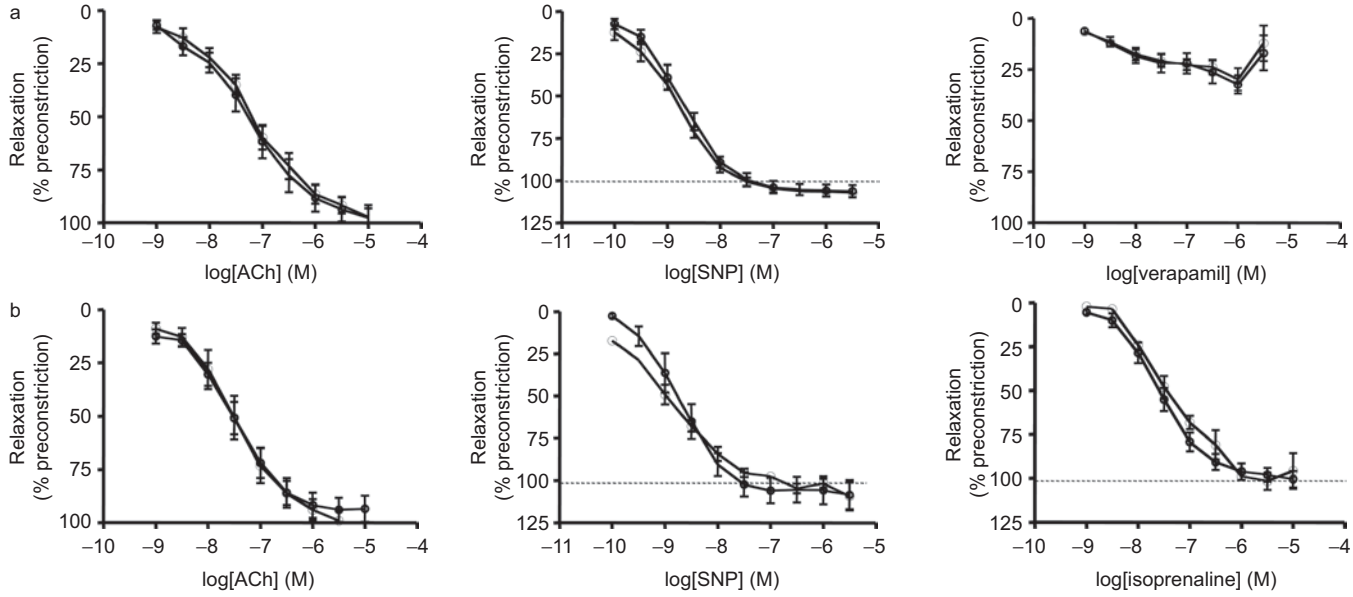

Figure 2. Effect of 4-week exposure to (A) diesel engine exhaust (PM_{DEE}) or (B) concentrated ambient particles near a busy roadside ($PM_{2.5}$ roadside) on *ex vivo* responses to vasodilator agents in rat aortic rings. Exposed groups (filled circles) and filtered air control (open circles). Values are shown as mean ± SE (ACh, $n = 5$–6; SNP, $n = 4$–6; isoprenaline/verapamil, $n = 4$–6). There were no significant differences between PM and control exposures ($P > 0.05$ for all).

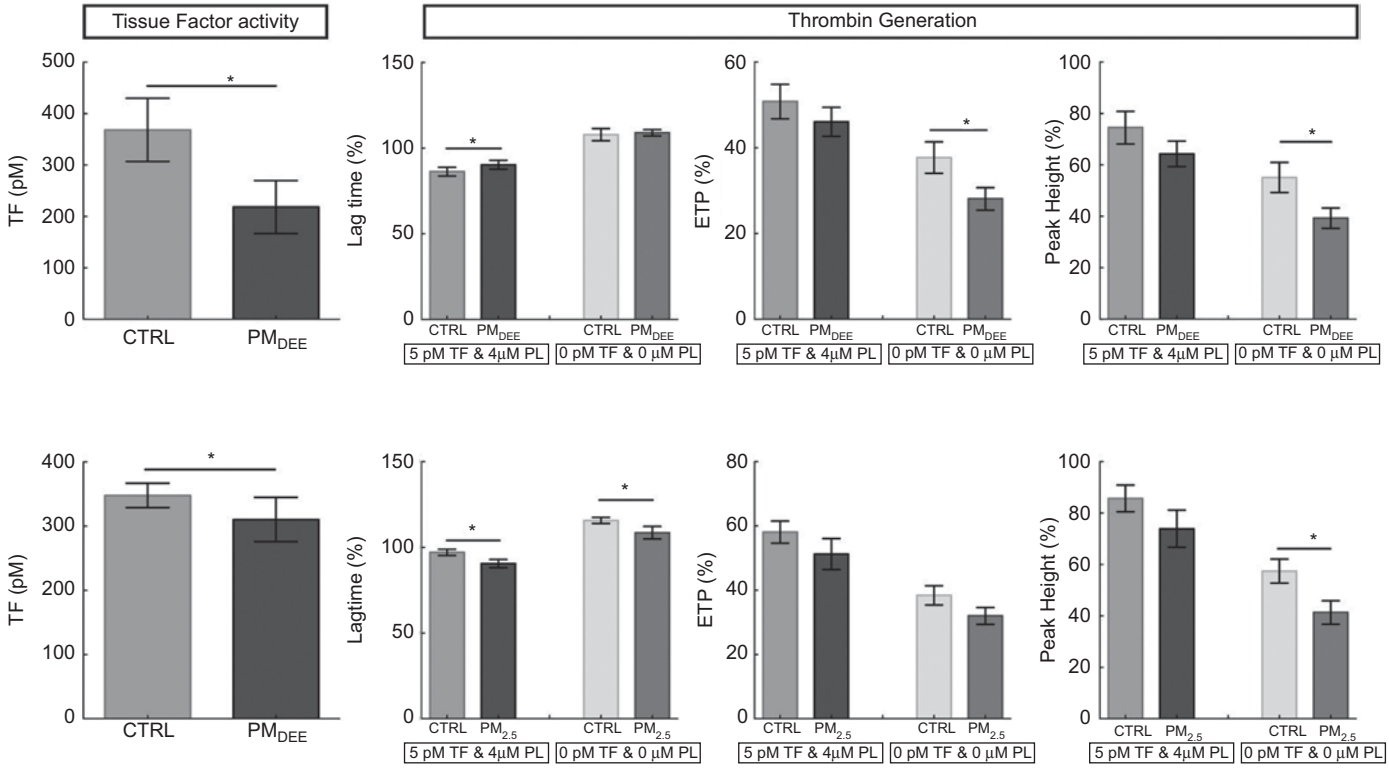

Figure 3. Tissue factor (TF) activity in and overall procoagulant activity of lung homogenate after 4 weeks exposure to diesel engine exhaust (PM_{DEE}: upper panels) or concentrated ambient particles near a busy roadside ($PM_{2.5}$ roadside: lower panels). Lung tissue-specific thrombin generation was performed in the presence of additional 5 pM TF and 4 μM phospholipids (5 pM TF and 4 μM PL) or in the absence of both TF and phospholipids (0 pM TF and 4 μM PL). Three parameters were derived from the obtained thrombin generation curves: lag time, defined as the time reaching 1/6 of the maximum peak thrombin; ETP, the endogenous thrombin potential or the area under the curve; and peak height, the maximum thrombin generated. Tissue factor activity is expressed as pM corrected for total protein content of 2.5 mg/ml in the lung homogenate. Thrombin generation parameters are expressed as percentage of normal human pooled, platelet-poor plasma, which served as an internal control. Bars indicate mean ± SEM of $n = 15$ animals per groups. *$P < 0.05$.

thrombin generation upon exposure to PM_{DEE} was confirmed by a decrease in peak height (control 55% ± 6% versus PM_{DEE} 39% ± 4%; $P = 0.018$; Figure 3). Furthermore, maximum thrombin generation given by the peak height was decreased after $PM_{2.5}$ exposure (control 57% ± 5% versus $PM_{2.5}$ roadside 41% ± 5%; $P = 0.020$; Figure 3) confirming the trend in attenuation of the ETP.

Discussion

Prolonged exposure to traffic-related PM at levels approximately 10 times higher than ambient levels, or exposure to specifically diesel engine exhaust, exerted only modest effects in relatively healthy rats. This was irrespective the fact that a mild inflammation was induced at the onset of exposure. Accumulation of particles within alveolar macrophages was observed in both $PM_{2.5}$ roadside exposures demonstrating that fine particulates are capable of reaching deep into the alveolar spaces. Biological changes were mainly of a cardiovascular nature, as shown by reduced WBC numbers, diminished levels of vWF protein, and reduced lung tissue thrombogenicity or procoagulant activity.

The fact that only very mild effects were detected in this study may be related to the adequately functioning host defense system of the rats. The animals were exposed to ozone (800 μg/m^3 for 12 h) prior to prolonged exposure to traffic PM, which was intended to cause significant, yet non-severe, pulmonary inflammation to compromise the defense system at the beginning of exposure to PM. Ozone is known to provoke damage of type I epithelial cells and increased permeability of the alveolar walls (Bhalla, 1999; Dormans et al., 1990). Previous studies in our laboratory (van Bree et al., 2001, 2002) under similar conditions as in the current study (12–24 h; 800 μg/m^3 ozone) resulted in a 2–3-fold increase protein levels in BALF, as well as a moderate influx of neutrophils (10–20% of total lavage cells). However, the inflammation induced in our study was rather mild, as only a slight (2.5%) increase in inflammatory cells was observed. On the other hand, a similar rise in lung permeability was found as reported previously. The difference in response might be caused by a difference in sensitivity between Fisher-344 rats used in the present study and the Wistar rats used previously. Nevertheless, ozone exposure was found to cause a similar degree of lung permeability to that found previously. This is important as an increase in permeability of the alveolar wall may assist in the translocation of particles from the lung into the circulation; one of the key mechanisms proposed to explain the systemic actions of inhaled particles (Geiser and Kreyling, 2010). Because accumulated particles within macrophages were observed in the present study, it seems plausible that translocation to the system circulation had taken place. Many epidemiological studies have claimed that, in particular, people with compromised airways are more likely to develop adverse health effects due to exposure to PM. This is in line with our observation that the rather healthy rats do not develop biological relevant adverse responses due to traffic-derived PM.

Using our diesel powered generator, a stable highly controlled test atmosphere was created that consisted of soot particles. The PM levels that were applied in this study can easily be detected in hot spots, such as road tunnels or at kerb sides of busy city streets. Ambient PM has been shown to have substantial spatial and temporal variation, both in terms of amount and physicochemical composition and that the contribution of secondary inorganic components although to play a very limited role in inducing toxicity (Schlesinger and Cassee, 2003) contributed on average ~25%. These factors might very well explain the intra- and inter-experimental variability observed for the two roadside experiments. The PM mass concentrations were higher than those applied in the PM_{DEE} exposure. On the basis of epidemiological associations that suggest a linear concentration–response relationship between PM and cardiorespiratory responses, it was assumed that PM mass concentrations would be predictive of the biological responses in the present study, however, this did not appear to be the case. Because the two roadside experiments led to higher, albeit distinctly different, average PM mass concentrations, according to the general assumption, any effect seen for PM_{DEE} should also be observed in the $PM_{2.5}$ roadside experiments. However, most parameters responding in the PM_{DEE} experiment were not affected by the PM roadside exposures, which implies that other factors than PM mass (i.e. PM size and chemical composition) affect the *in vivo* responses. Indeed, previous studies by our group and others (Schwarze et al., 2006; Gerlofs-Nijland et al., 2007, 2009b) suggested that factors such as chemical composition are driving the toxicity. Another important difference between the PM_{DEE} exposure and the exposure to roadside $PM_{2.5}$ is the higher gaseous pollutant concentrations for the exposure to diesel engine exhaust. As the PM_{DEE} gaseous components were not exceeding limit values as defined by American Conference of Governmental Industrial Hygienists (ACGIH, 1991), we can assume that these could not explain the observed vascular responses.

One more variable between the PM_{DEE} and $PM_{2.5}$ roadside exposures is the particle number concentration, with substantially higher numbers for the PM_{DEE} experiment. Diesel engine exhausts are dominated by particles of approximately <100 nm, which are also referred to as ultrafine particles. Several authors have suggested that ultrafine particles have adverse effects on the cardiovascular system (Delfino et al., 2005; Schulz et al., 2005; Knol et al., 2009). Therefore, it may very well be that in our $PM_{2.5}$ roadside experiments, in which the numbers of ultrafine particles were lower than the PM_{DEE}, the number of ultrafines has played a more dominant role than PM mass.

Another explanation for the observed limited responses might be the development of adaptation caused by the long exposure duration. It is generally known that various biological markers have their optimal effect at different time points. Moreover, some markers like MAPKs may be activated over time in a multi-phasic way, i.e. even baseline levels vary from day to day (Thrane et al., 2001; Chen et al.,

2003). Reduced vWF protein levels were already observed 6 days after exposure to PM_{DEE} and reached significance after 4 weeks. Measuring at the different time points might also implicate that changes in adaptative pathways are observed. This may explain some of the contradictory observations of other groups increased vWF levels to traffic-related PM (O'Neill et al., 2007; Yue et al., 2007), compared to a decrease in vWF in association with air pollutants (Carlsten et al., 2008; Hildebrandt et al., 2009). Elevated plasma vWF levels may imply an increased risk for thrombosis (Franchini and Mannucci, 2008), therefore, the time course of thrombotic responses may also vary between acute, subchronic, or prolonged exposure to air pollutants. Adaptation pathways to chronic exposures present in healthy animals may be impaired in disease; therefore, experiments are currently underway in our laboratory examining the actions of PM_{DEE} on the cardiovascular system in a model of atherosclerosis.

The impaired lung procoagulation activity after prolonged exposure to traffic-related PM is supported by the reduced lung TF activity in conjunction with reduced lung tissue thrombin generation. In general, acute exposure to air pollution is associated to increased hypercoagulability shown by shorter prothrombin time (lag time) and elevated plasma thrombin generation (Baccarelli et al., 2007; Bonzini et al., 2010). However, these epidemiological studies provide insight in the plasma hypercoagulable state after acute exposure to air pollution, whereas impaired tissue procoagulant activity might also indicate an adaptive defense mechanism.

In our clinical studies, we have shown that a 2-h exposure of healthy volunteers to Edinburgh $PM_{2.5}$ had no effect on vessel wall function as determined by forearm plethysmography (Mills et al., 2008). On the other hand, diluted diesel engine exhaust attenuated responses to the endothelium-dependent vasodilator ACh and the endothelium-independent vasodilator SNP, but not to the NO-independent vasodilator, verapamil. Previously, we have demonstrated in an animal experiment that similar responses occurred 4 h after acute exposure to various PM samples by intratracheal instillation (Bagate et al., 2004). In addition, diesel engine exhaust particles directly inhibit vascular relaxation to endothelium-dependent vasodilators (Miller et al., 2009). Therefore, we assumed that prolonged exposure to PM_{DEE} and not roadside $PM_{2.5}$ was associated with cardiovascular impairment. However, no signs of impairment were observed after prolonged exposures applied in the three experiments presented in this paper.

In the present study, we noted a decrease of WBC in the PM_{DEE} as well as in the second $PM_{2.5}$ roadside experiment. Similar observations have been made in rats after acute exposure to traffic-related PM (Gerlofs-Nijland et al., 2005; Kooter et al., 2006). In human studies, Frampton and co-workers (2002) noted that NO_2 exposure resulted in reduced lymphocytes that migrate to the lung, as increased lymphocyte numbers were found in the respiratory system. Recently, changes in differential WBC was reported in patients with chronic pulmonary disease related to ambient air pollution exposure (Brüske et al., 2010). Although the biological significance and impact is still not clear, it seems that reduced circulating WBCs are related to increased exposure to air pollutants.

Freshly generated PM_{DEE} induced mild cardiovascular responses (impaired coagulation) but no respiratory effects were seen in relatively healthy rats. Also no biological relevant changes were detected after exposure to ambient roadside $PM_{2.5}$. The overall analysis of the results did not support the hypothesis that PM mass concentrations are linear related to health effects. In contrast to common belief, prolonged exposure to traffic-related PM in healthy animals may not be detrimental due to various biological adaptive response mechanisms. It could be speculated that vulnerability of humans to acute or repeated exposure to PM may be primarily dependent on the presence of comorbidity such as coronary heart disease. We conclude that prolonged although not chronic exposures in healthy animals have very limited impact on pulmonary and cardiovascular function. Further studies are needed in animals with established disease (such as more extensive pulmonary inflammation or developed cardiovascular disease) to reveal the influence of susceptibility on air pollution–induced toxicity.

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Declaration of interest

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